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(54) Title: BIOCOMPATIBLE PROSTHETIC DEVICES		
(57) Abstract The present invention provides a prosthetic device comprising an arrangement of collagen threads, each thread formed from a solution of collagen molecules. The arrangement of the threads provides a scaffold for the infiltration and population of host connective tissue cells that eventually supplement or replace the device with natural tissue.		

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BIOCOMPATIBLE PROSTHETIC DEVICES

FIELD IN THE INVENTION

The present invention is in the field of implantable medical devices and prosthesis. More particularly, the present invention is directed to prosthetic devices for use in, e.g., ligament, repair and replacement, hernia repair, blood vessel replacement, and to methods of their fabrication and use.

BACKGROUND OF THE INVENTION

One of the most important attributes of living organisms is their capacity for self-repair. Several mechanisms have evolved to achieve this, including wound healing, compensatory growth and epimorphic regeneration [J. Gross, Regeneration versus repair, pp. 20-39 (1992), In: L.K. Cohen, R.F. Diegelman and W.J. Lindblad (eds.), *Wound Healing: biochemical and clinical aspects*, W.B. Saunders, Philadelphia]. Although all mammalian tissues and organs (with the possible exception of teeth) are capable of some degree of repair, mammals have unfortunately lost the ability to faithfully regenerate severely damaged body parts [J. Gross, *supra* (1992)]. In an attempt to overcome this deficiency, numerous synthetic devices have been developed using organic polymers, with the intention that the implants be biologically inert, and yet function for the lifetime of the recipient. However, not only is biological inertness

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apparently impossible, but the interaction between biomaterial and the surrounding living tissue can actually contribute to the long-term success of the implant [J. Kohn, *Med. Dev. Technol.*, 1:34-38 (1990)]. The science of Tissue Engineering has arisen to exploit this biological reality.

Tissue engineered grafts, implanted with or without living cells, are designed not only to perform an immediate physical function, but equally important to guide and encourage appropriate tissue formation within the graft. The new tissue forms as the original graft material biodegrades, thus creating a permanent function analog of the original tissue. This approach is presently under investigation to repair such structures as skin [J.F. Burke, *Ann. Surg.*, 194:413-428 (1991); G.G. Gallico, III, *N. Engl. J. Med.*, 311:448-451 (1984); J.F. Hansbrough, *J. Am. Med. Assoc.*, 262:125-130 (1989); J.F. Hansbrough, *J. Burn Care Rehabil.*, 13:519-528 (1992); D. Heimbach, *Ann. Surg.*, 208:313-320 (1988); N.L. Parenteau, *J. Cell Biochem.*, 45:245-251 (1991); I.V. Yannas, *Science*, 215:174-176 (1982); I.V. Yannas, *Proc. Natl. Acad. Sci. USA*, 86:933-937 (1989)], blood vessels [R.M. Carr, et al. *Mat. Res. Soc. Symp. Proc.*, 252:175-182 (1992)], cartilage [C.P. Vacanti, et al., *J. Plast. Reconstr. Surg.*, 88:753 (1991); S. Wakitani, et al., *J. Bone Joint Surg.*, 71:74-80 (1989)], and tendons [Y.P. Kato, *J. Bone Joint Surg.*, 73A:561-574 (1991)].

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The biomaterials used to produce such remodelable grafts are the focus of a great deal of study. Two families of materials are being evaluated by a number of investigators: biological components of the extracellular matrix (ECM), such as the collagens and proteoglycans, and synthetic, non-biological materials. Biologically derived materials are advantageous in that they contain information that facilitates cell attachment and function, whereas synthetics may not interact with cells in the desired manner [R. Langer, *Science*, 260:920-926 (1993)]. Investigators are presently attempting to alter synthetics by coupling peptide sequences recognized by cell adhesion proteins such as the integrins [J.A. Hubbell, *Ann. NY Acad. Sci.*, 665:253-258 (1992); H.B. Lin, *et al.*, *Biomaterials*, 13:905-914 (1992)].

The original idea that the ECM is merely an inert supporting material on which the cells reside is now generally regarded as false [E.D. Hay, *et al.*, *Cell Biology of Extracellular Matrix*, 2nd edition (1991), Plenum Press, New York; C. Nathan, *J. Cell Biol.*, 113:981-986 (1991)]. Cells continue to interact with the many components of the ECM, which together serve the functions of adhesive, biomaterial, filter, receptor, signal and text [C. Nathan, *supra* (1991); R.L. Trelstad, *Textbook of Rheumatology*, pp. 35-57 (1993) 4th edition, W.B. Saunders, Philadelphia]. Therefore, it seems reasonable to suppose that the complex interactions between cells and the ECM are such that biologically derived

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implants will continue to provide stimuli to guide remodeling, that synthetics cannot, unless they are modified to such an extent that they become essentially identical to the natural molecules they are attempting to mimic.

Although Type I collagen has been utilized as a biomaterial for over 50 years, such implants have not generally exploited the body's ability to remodel an implant. On the contrary, implants were intended to be permanent, and the manufacturing processes used to produce these devices either utilized partially degraded, enzyme-extracted collagen, or stabilized the collagen by crosslinking it with cytotoxic glutaraldehyde or chromium salts [M. Chvapil, Industrial uses for collagen., In: D.A.D. Parry and L.K. Creamer (eds.), Fibrous proteins: scientific, industrial and medical aspects, (1979) Academic Press, London.; M. Chvapil., Int. Rev. Connect. Tiss. Res., 6:1 (1973); K.H. Stenzel, et al., Ann. Rev. Biophys. Bioeng., 3:231-253 (1974)], or else assembled the collagen into non-natural polymeric structures such as films and sponges [J.F. Burke, *supra* (1981); M. Chvapil, *supra* (1979); M. Chvapil, *supra* (1973); D. Heimbach, *supra*, (1988); A. Rubin, J. Macromol. Sci. Chem., A3:113-118 (1969); K.H. Stenzel, *supra* (1974); I.V. Yannas, *supra* (1982); I.V. Yannas, *supra* (1989)]. Type I collagen has been used in the production of a living skin replacement (Graftskin™, Organogenesis, Inc. See, e.g.,

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U.S. Patent No. 4,485,096), presently in human clinical trials. Although the collagen used in the living skin replacement is reassembled into native banded fibrils, they are shorter than normal, and the resulting material does not have the mechanical properties of actual dermis. Since prosthetics that must function under significant loads, such as ligaments and tendon replacements and in hernia repair, require significant mechanical strength, alternatives are being sought.

Connective tissues derive their tensile strength and physical character mainly from the three-dimensional assembly of long, intertwined crosslinked collagen fibrils and fibers. Reconstituted collagen fibers have been used for over 50 years as suture material [F.O. Schmitt, *Ann. Rev. Biophys. Biophys. Chem.*, 14:1-22 (1985); K.H. Stenzel, *supra* (1974)]. These fibers have either been discontinuous [M. Chvapil, *supra* (1979); Y.P. Kato, *Biomaterials*, 11:169-175 (1990)] or continuous methods [Y.P. Kato, *supra* (1990); F. Rodriguez, *Polymer News*, 9:262-265 (1984)]. Although it has been postulated that such fibers could be knitted or woven into fabrics [M. Chvapil, *supra* (1979); F. Rodriguez, *supra* (1984); A.L. Rubin, *supra* (1969)]; the production of such fabrics has not been reported. The production of such fabrics require large amounts of material. Typical runs on industrial knitting and braiding machinery requires a minimum of 5 km of thread. For this practical reason, there has apparently

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been little investigation into the clinical applicability of such biological textiles.

One important area of tissue engineering is the development of material to facilitate wound repair such as hernia, ulcer, and damaged blood vessels.

Another important area for tissue engineering is the development of a material to permanently replace a damaged ligament or tendon. The tendon or ligament replaced most commonly is the anterior cruciate ligament (ACL) of the knee. Most often, athletic injuries (football, skiing) are the cause. Once this particular ligament is torn, healing does not occur by itself as it may in other knee ligaments (e.g., the medial collateral ligament) primarily because apposition of the torn ligament stumps is impossible due to elastic contraction.

The first choice material for replacing a damaged ACL is a patellar tendon autograft. Typically, a slice of the patellar tendon about one-third of its width is removed from the same knee joint and placed in the anterior cruciate position; fixation is usually accomplished by precise drilling into the femur and tibia, placing the graft in between, and potting the ends in bone cement within the drilled holes.

From an FDA standpoint, autografts are not regulated medical devices, so they are not subject to any of the manufacturing and marketing laws governing synthetics. As such, the patellar tendon is free material available for use to the surgeon, making it an attractive ACL replacement

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option. In addition, autografts do not have the problem of immunological rejection, since the replacement tissue is from the patient's own body. However, harvesting the required tissue causes needless additional joint trauma. Moreover, the patellar tendon is weakened during the remodeling process and may then be damaged. Ultimately, the failure rate of patellar tendon autografts is high because they are slow to revascularize, and often elongate to the point where knee loads are no longer supported.

Cadaveric ACL replacements or patellar tendon grafts have been considered as an option to autografts. However, cadaveric autografting presents a significant immunological problem for the recipient; further, they have the potential for transmitting any of a number of viral pathogens.

After the failure of a patellar tendon autograft, surgeons consider a prosthetic device for ACL replacement. Advances in materials science have produced grafts which are very strong and durable, even surpassing the natural strength of the original ACL. The primary materials considered for ACL replacement grafts are synthetic polymers, carbon fibers, and collagen.

Synthetic grafts, generally polyethylene terephthalate (Dacron, manufactured by Stryker and Howmedica) or polytetrafluoroethylene (Teflon, made by Gore-Tex), and even carbon fiber grafts (DuPont), are very tough. They may be twice as strong as natural ACL tissue; they endure up to 10^7 bending cycles without failing. However, bench tests cannot simulate the internal milieu of the knee joint. Although these grafts start out strong enough to bear the required loading, these materials are far from inert; they are subject to fatigue, abrasion and immunological attack once

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implanted. Problems of chronic inflammation and abrasion persist and worsen with time, until mechanical failure occurs, necessitating reoperation.

Recently, ACL grafts made from reconstituted collagen fibers have been reported. U.S. Patent No. 5,171,273 disclosed a collagen graft comprising synthetic collagen fibers embedded into a loose uncrosslinked collagen matrix. The patent disclosed the disassociation of bovine corium (dermis) to obtain "insoluble collagen" which is the starting material for the fibers. This solution is a suspension of fragments of native banded, fibrillar bovine Type-I collagen, and is believed to contain small amounts of other tissue proteins.

U.S. Patent No. 5,263,984 disclosed a prosthetic ligament comprising filaments formed of fibrils or short pieces of native polymeric connective tissues such as collagen. The starting collagen material in the case was also insoluble.

Neither of the above-discussed devices mimic the chemical and organization structure of natural collagen. Such a device would be desirable.

It would also be desirable to have a prosthetic device that combines the advantages of the available devices and also reduces the incidence of graft problems.

SUMMARY OF THE INVENTION

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The present invention provides a prosthetic device comprising an arrangement of collagen threads, each thread being formed from a solution of collagen molecules. In one embodiment, these devices are useful in regenerating ligamentous tissue *in vivo*. The arrangement of the threads provides a scaffold for the infiltration and population of host connective tissue cells that eventually supplement or replace the device with natural tissue.

The enhanced biocapability of the device reduces the host inflammatory response, while at the same time avoiding the additional trauma of harvesting host tissue. Because the device is augmented or replaced with living host tissue having the same cell population, vascular supply, and self-renewing properties as the original tissue, no abrasion particles are formed as typically occurs with synthetic grafts.

In one embodiment, the threads of the device are arranged to form a bundle. In another embodiment, the threads are arranged to form a braid. In certain other embodiments, the threads are arranged in the form of a knitted fabric.

The threads may be crosslinked. The crosslink may be formed by any known crosslinking agent including, for example, aldehydes (e.g., cyanamide, 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride (EDC)). A preferred chemical agent is EDC. Larger devices may be formed by crosslinking a number of smaller devices.

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In certain embodiments, the device may comprise a mixture crosslinked and non-crosslinked threads. In a preferred aspect of such an embodiment, the non-crosslinked threads are arranged on the outside of the device.

Preferred collagen threads for use in the prosthetic devices of the present invention have an ultimate tensile strength of greater than about 1 MPa for non-crosslinked threads and greater than about 45 MPa for crosslinked threads. Preferably, the threads have an ultimate tensile strength from about 1.0 MPa to about 100 MPa.

The present invention further provides a prosthetic ligament. In a preferred embodiment, the ligament is a prosthetic anterior cruciate ligament.

The present invention further provides a prosthetic device comprising a knitted fabric formed from collagen threads. In a preferred embodiment, the fabric is warp knitted. In accordance with the present invention, the extensibility and porosity of such a knitted fabric can be controlled by varying the knit angle, as well as the diameter of threads.

Further, the invention includes a method of regenerating the ligamentous tissue *in vivo*. This method includes providing a prosthetic ligament of the invention and implanting it into a joint by known surgical procedures.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic of a collagen threadmaking apparatus.

Figure 2 shows a tricot-knitted collagen fabric (bar = 3 mm).

Figure 2A is a single bar, open mesh fabric;

Figure 2B is a double bar, open mesh fabric. The pattern of each bar is the same as in Figure 2C;

Figure 2C is another double bar fabric. The two bars have different patterns.

Figure 2D is a triple bar knit, designed for high bulk and low extensibility.

Figure 3a and 3b are light micrographs of collagen threads (bar = 200 μm) using normal illumination (Figure 3a) and plane polarized light (Figure 3b), showing a strong birefringence pattern.

Figure 4a and 4b show the morphology of collagen threads. Figure 4a is a scanning electron micrograph (SEM) of the broken end of a dry collagen thread (bar = 20 μm). Figure 4b is a transmission electron micrograph (TEM) of a longitudinal section through a collagen thread stained with lead citrate and uranyl acetate. Collagen fibrils (cf) with characteristic banding patterns were seen on the surface. The center of the thread contained fine filaments (bar = 200 nm).

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Figures 5a-5b are photographs of collagen braids and bundles (ruler in cm). Figure 5a (bottom) is a 32-ply braid, fabricated in two steps. A 8-ply braid was formed on a braiding machine and this material was then re-run through the machine as a 4-ply. Figure 5a (top) is a 384-ply braid, fabricated in four steps. An 8-ply braid was formed on a braiding machine and this material was then re-run twice through the machine as a 4-ply, before finally being braided 3-ply by hand. Figure 5b (bottom) is a 50-ply bundle fabricated by winding collagen thread around a 20 cm fixture. Figure 5b (top) is a 600-ply bundle fabricated by winding collagen thread a 20 cm fixture. The bundle was then held by 16-ply collagen braid in order to form the end loop.

Figure 6a and 6b are graphs showing collagen thread mechanical properties after EDC crosslinking. Figure 6a is shrinkage temperature measured by heating a loop of collagen thread loaded with 2.5g. The temperature at which the loop length decreased by 10% was taken as the shrinkage temperature. Figure 6b is tensile strength of collagen threads wetted in PBS was measured on a 50 mm gauge length and at strain rate of 50% per minute.

Figures 7a - 7c are photographs showing the morphology of the collagen thread ligament prosthesis implanted as a canine anterior cruciate ligament and explanted at 12 weeks. Figure 7a is a photograph adjacent to the fixation staple. Inflammatory cells are found around the intact collagen threads (t). (bar = 300 μ m). Figure 7b is a photograph of a longitudinal section in the mid-substance of the explant taken between the femoral and tibial insertion points. New collagenous tissue (c) is seen with embedded

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mesenchymal cells (m). (bar = 300 μ m). Figure 7c is a photograph at the bone interface. Collagenous tissue (c) is seen interdigitating with the articular cartilage (ac). (bar = 300 μ m).

Figures 8a and 8b are transmission electron micrographs of the collagen thread ligament prosthesis implanted as a canine anterior cruciate ligament and explanted at 12 weeks. Samples were strained with lead citrate and uranyl acetate. Figure 8a is the ligament prosthesis explanted at 12 weeks. (bar = 500 nm). Figure 8b is the contralateral control ligament. (bar = 500 nm).

Figures 9a and 9b are histograms of collagen fibril diameter distributions seen in remodelled collagen thread ligament prosthesis implanted as a canine anterior cruciate ligament and explanted at 12 weeks. Figure 9a is a collagen thread prosthesis. Figure 9b is a contralateral control ligament.

Figure 10 is a morphology of knitted collagen fabric implanted in a full-thickness abdominal defect in a rat and explanted at 3 weeks (bar = 1 mm). Intact collagen threads (t) (which have shrunk during histological processing) can be seen surrounded by inflammatory cells (i) and fibroblasts which have laid down new connective tissue (c).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a biocompatible prosthetic device useful as an implant for repairing damaged tendons and ligaments and other structures as well as in methods of hernia repair, blood vessel replacement,

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prolapse support and chest wall reconstruction after trauma or tumor resection. In one preferred embodiment, the ligament replaced is the anterior cruciate ligament in the knee joint of mammals.

The devices of the present invention comprise an arrangement of collagen threads formed from a solution of collagen molecules, that mimic the chemical and organization structure of natural collagen. The device, once implanted, provides a scaffold for the infiltration and population for host connective tissue cells that eventually supplement or replace the device with natural tissue, thus allowing it to perform its natural functions.

Techniques useful for inserting autographs or synthetic grafts may be used to implant the device of the present invention. Such techniques include, for example, the "over-the-top technique" [R.D. Montgomery, et al. *Clin. Orthop. Rel. Res.*, 231:149-153 (1988)]

In accordance with the present invention, acid extraction rather than enzyme extraction is used to produce a collagen solution. Enzyme extraction (pepsin) removes the telopeptide regions from the ends of the collagen molecule; such collagen preparations could not be used for thread production in the preferred thread making system. Similarly, pepsin extracted collagen has been shown to produce fibroblast contracted collagen lattices which are twenty times weaker than similar lattices produced from acid extracted collagen [E. Bell, *INSERM*, 177:13-28 (1989)]. Acid solubilized collagen can be formed using techniques known to the skilled artisan. See, U.S. Patent 5,106,949 and WO93/06791.

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Preferred details as to the method of forming acid solubilized Type I collagen are given in the Examples below. Briefly, one preferred method of preparing collagen for use in preparing thread for use in the present invention comprises the steps of: (a) washing finely-divided tendon in sodium chloride/sodium phosphate buffer; (b) extracting collagen from the washed tendon with acetic acid and a gentle mechanical action; (c) precipitating the acid-extracted collagen obtained in step (b) with sodium chloride; and (d) recovering the precipitated collagen.

The formation of collagen threads using acid solubilized Type I collagen is described in published PCT application WO93/06791, which is incorporated herein by reference. Further details set forth below.

One preferred method of making collagen threads for use in the present invention comprises:

(a) extruding a solution comprising collagen into a dehydrating agent, the dehydrating agent having a higher osmotic pressure than that of the collagen solution and a pH from about 5 to 9; and

(b) maintaining the dehydrating agent under conditions to enable collagen thread formation.

In another preferred method, the method of making collagen threads further comprises rinsing the formed thread in a buffer to provide additional flexibility. This optional step is particularly useful in applications wherein the collagen thread will be knitted or woven.

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In some instances, it is desirable to crosslink the collagen thread in order to improve its strength, particularly its wet strength. Accordingly, in yet other preferred embodiments of the present invention, the collagen thread is crosslinked. Although crosslinking may be carried out without rinsing the thread, in particularly preferred embodiments the thread is rinsed and dried before crosslinking is carried out.

In yet other preferred methods of the present invention, banded collagen threads are produced, at least in part, by selecting the appropriate dehydrating agent system.

Collagen for use in the present invention may be obtained from any suitable source. Typical sources include skin and tendons. Many procedures for obtaining and purifying collagen, typically involving acid or enzyme extraction, are known to practitioners in the art and may be used to prepare collagen for use in the present invention. A preferred collagen composition for use herein is obtained from a novel source, the bovine common digital extensor tendon, and by a novel extraction method, both as disclosed in copending U.S. Patent No. 5,106,949 the disclosure of which is incorporated herein by reference. Although both monomers and mixtures of monomers and higher ordered collagen polymers, e.g., dimers up to and including fibrils, can be used in the practice of the present invention, monomers are preferred for many applications.

Collagen solutions for use in the present invention are generally at a concentration of about 2 to 10 mg/ml, preferably, from about 4 to 6 mg/ml, and most preferably at about 4.5 to 5.5 mg/ml and at pH of about

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2 to 4. A preferred solvent for the collagen is dilute acetic acid, e.g., about 0.05 to 0.1%. Other conventional solvents for collagen may be used as long as such solvents are compatible with thread formation and the desired properties of the collagen thread. These collagen solutions may contain optional components known to those of ordinary skill in the art, such as neutral and charged polymers, including but not limited to, polyvinyl alcohol and hyaluronic acid.

The collagen solution is preferably degassed or debubbled before extrusion into a dehydrating bath. This may be accomplished, for example, by centrifugation, as well as other methods well known to practitioners in the art.

The dehydrating bath comprises a dehydrating agent having a higher osmotic pressure than that of the collagen solution, preferably higher than about 500 mOsm, and a pH from about 5 to 10, with a pH of about 7 to 9 being preferred. Preferred dehydrating agents include water soluble, biocompatible polymers such as DEXTRAN® and polyethylene glycol (PEG). In preferred embodiments, the dehydrating agent is dissolved in a buffer such as sodium phosphate or sodium borate. One preferred dehydrating bath for use in the methods of the present invention comprises about 20 to 30% by weight PEG in phosphate buffer.

Native-banded collagen fibrils are typically formed within the collagen threads, when the dehydrating bath comprises sodium phosphate at 0.1 to 0.5 M. It has been found that borate buffer inhibits the formation of

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native-banded fibrils. While not wishing to be bound by theory, it is believed that borate may inhibit band formation by binding to the collagen.

In embodiments wherein it is desired to impart additional flexibility to the thread, e.g., where the thread is to be knitted or woven, the collagen thread may be rinsed. A preferred rinsing bath for use in the present invention comprises phosphate buffered saline ("PBS") having a phosphate concentration of about 0.001 to 0.02 M and a NaCl concentration of about 0.05 to 0.1 M. In a particularly preferred embodiment, a phosphate buffered saline solution is prepared as described hereinafter in Example 1. The pH of the rinsing bath is kept above a pH of about 5, to prevent the thread from over hydrating. A preferred pH range is from about 6 to about 8.

In another preferred embodiment, the collagen thread is crosslinked to increase the strength, e.g., the wet tensile strength, of the thread. This can be accomplished by any number of methods known to those of ordinary skill in the art, including lyophilization, u.v. irradiation, or contact with an aldehyde such as glutaraldehyde. Carbodiimides such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) have also been used to crosslink collagen thread, but such crosslinking occurs at a slower rate than with glutaraldehyde.

For the purpose of description only, the methods for use in producing threads will be illustrated by preparing collagen threads by means of the apparatus shown in Figure 1. It will be understood to those skilled in the

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art that the methods of the present invention are not limited to the apparatus shown.

Referring to Figure 1, there is shown a schematic diagram of one form of an apparatus which may be used for producing collagen threads in accordance with the present invention. As shown in Fig. 1, the apparatus comprises means for extruding the collagen solution 1, dehydrating bath 10, rinsing bath 20, means for drying the collagen thread 30, and means for collecting the collagen thread which includes uptake spool 40 and a driver, not shown, for the uptake spool 40.

As shown in Fig. 1, means 1 for extruding a collagen solution includes a syringe pump 2, a syringe 3, leader tubing 4, and a blunt needle 5. The dehydrating bath 10, includes dehydrating trough 11, dehydrating agent 12 and recirculating pump 13. The rinsing bath 20 includes a rinsing trough 21 and rinse liquid 22. The means for drying the collagen thread 30 includes a drying cabinet 31, pulleys 43 to 47, and a heated blower 32. The thread is carried through and out of the apparatus by means of a series of pulleys 43 to 47 and uptake spool 40, driven by an uptake driver, not shown.

In one preferred embodiment, a solution of collagen at 5 mg/ml in 0.05% acetic acid is degassed, loaded into the syringe 3, and connected to the leader tubing 4 and needle 5. The syringe 3 is placed in position relative to syringe pump 2 so that the pump 2 can act on the syringe 3 to extrude the collagen solution. The leader tubing 4 and needle 5 are placed in the dehydrating bath 11 under the surface of dehydrating agent 12.

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Although a syringe pump and syringe are used to illustrate the extrusion of collagen in accordance with the present invention, those of ordinary skill in the art of polymer extrusion will recognize that extrusion can also be carried out by use of other conventional devices.

Preferred materials for the apparatus described are compatible with thread formation, the desired properties of the thread and the materials used in thread formation, e.g., acetic acid at about 3 pH. In some instances the apparatus must be capable of sterilization.

The thickness of the extruded collagen thread is determined in part both by the rate of infusion of the collagen solution into the dehydration bath and flow rate of the dehydrating agent in the trough 11. The syringe pump 2 is set to extrude the collagen solution at a rate which is typically between about 2.0 to 3.5 ml/min., depending upon the collagen solution. Dehydration trough 11 is configured to provide sufficient capacity for the desired volume of dehydrating agent 12. Dehydration trough 11 is constructed of a material which is compatible with collagen and the reagents used. Such materials include PVC and polycarbonate. The dehydration trough 11 is provided with a recirculating pump 13 to recirculate the dehydrating agent 12. The rate of extrusion of the collagen solution and the rate of circulation of the dehydrating agent 12 and the length of trough 11 are selected to provide the desired minimum residence time in the dehydrating agent 12.

The flow of the circulating dehydrating agent 12 draws the thread. When a collagen thread 14 of sufficient length has been formed, the

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leading end of the thread is removed from the dehydration trough 11, and transferred into the rinsing trough 21.

When enough slack is generated, the thread is moved through the rinsing trough 21, disposed pulley 43 of the drying cabinet 30. A heated blower 32 is activated and the collagen thread is disposed on pulleys 44 to 47 and eventually on uptake spool 40. The drying temperature in the chamber is typically about 30°C to 45°C, more preferably 43°C. The speed of uptake spool 40 is controlled by an uptake driver, not shown, and adjusted so that the thread emerges dry to the touch from drying cabinet 30.

In some preferred embodiments, the collagen thread is crosslinked to increase its wet tensile strength. In one particularly preferred embodiment, the thread is removed from the uptake spool 40 after drying and then crosslinked by pulling the thread through a solution of 2% glutaraldehyde. By way of comparison, the wet strength of uncrosslinked collagen threads in accordance with the present invention has been found to be about 5-25 g, whereas the wet strength of crosslinked thread was about 50-150 g. If the thread is not crosslinked, it has been found that the wet strength is generally about 1-10% of the dry strength, whereas if the thread is crosslinked, the wet strength is about 60 to 100% of the dry strength.

The collagen threads prepared in accordance with the present invention may have a collagen concentration of about 300 to 600 mg/ml.

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When collagen produced by the methods of U.S. Serial No. 07/407,465, *supra.*, is used to prepare threads in accordance with the present invention, superior threads are obtained as compared to use of, e.g., commercially available pepsin extracted collagen (Pentapharm). For example, a collagen thread prepared in accordance with the present invention from such pepsin extracted collagen had a wet strength of only 2g, whereas thread produced using collagen of U.S. Serial No. 07/407,465 had a wet strength of 15g.

The methods of the present invention have been used to produce collagen threads of about 50 μm to 250 μm in diameter. However, threads having diameters outside this range can be produced if desired for certain applications. Typical physical properties of threads produced in accordance with the methods of the present invention are given in Table I below:

TABLE I:

Denier	75 - 90
Dry Strength (γ)	220
Elongation at break	20
Tenacity	2 - 3
% Moisture	30
Wet strength uncrosslinked (g)	15
Crosslinked (g)	180

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Collagen produced in accordance with U.S. Serial No. 07/407,465 was used to produce collagen threads in accordance with the present invention (Thread B). In Table II below, typical properties of Thread B determined using standard methodologies are compared with the reported properties of threads produced in Kato and Singer, *supra*. (See page 171, Table 1). (Thread A). Thread B was soaked in PBS in accordance with the teachings of Kato and Singer.

TABLE II: MECHANICAL PROPERTIES OF CONTINUOUS COLLAGEN THREADS AFTER SOAKING IN PBS

	<u>Thread A</u>		<u>Thread B</u>	
	Non XL	Glut XL	Non XL	Glut XL
Ultimate tensile strength (MPa)	0.8 \pm 0.2	37 \pm 7.9	1.7 \pm 0.6	70 \pm 7.0
Ultimate strain (%)	38 \pm 4.9	17 \pm 3.0	30 \pm 10	45 \pm 10
Modulus (MPa)	3.6 \pm 0.8	270 \pm 69	5.7 \pm 2.0	134 \pm 13
Load at Break (gm)	1.2 \pm 0.3	14 \pm 2.5	11 \pm 3.9	167 \pm 9.6
Swelling (%)	165 \pm 16	24 \pm 9.9	390 \pm 35	63 \pm 9.1

It can be seen that the collagen threads for use in the present invention have superior properties.

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Several factors play key roles in making collagen thread successful. The collagen is preferably substantially without bubbles because air bubbles present in the solution cause weak spots and resultant threads. Although leaks can be repaired by knotting the two ends of the threads, it is desirable to avoid such repairs. The coagulation bath speed, which determines the amount of draw at the needle orifice, must be steady, or it can drastically affect denier. If the bath is too fast, the thread may be made so finely as to break from the orifice before gelling; if too slow, the collagen infusion will not form a thread, but an amorphous gel-like mass. The flow in the bath must be kept substantially laminar, in order to prevent turbulence from tangling the thread beyond repair. At this point in the development of the process, thread uptake rate determines the production rate. The uptake is preferably fast enough to accomplish about 100% draw within the cabinet, yet allow enough residence time to dry completely, or the thread tensile properties are compromised. Present production rates are 20 times faster than previous published rates for preparation of a continuous collagen thread [Y.P. Kato, *supra* (1990)].

Breaks should be repaired swiftly by knotting the two ends, so that uptake (and correct draw) can be resumed as soon as possible. Subsequent winding, twisting, braiding, warping and/or knitting operations require maximum consistency of denier and strength. Flaws, such as knot tails, can be trimmed to a manageable size if not too frequent. Plying (either by twisting or braiding) not only can increase the strength of the yarn being worked, but also is good protection against breakage at a knot.

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Once formed, the collagen threads or a portion of the threads can then be crosslinked to increase the strength of the device. This can be accomplished by any number of methods known to one of ordinary skill in the art, including, for example, lyophilization, U.V. irradiation, or contact with an aldehyde such as glutaraldehyde or carbodiimides such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC).

Aldehyde crosslinking was accomplished by exposing the threads to glutaraldehyde vapor overnight, or by soaking the threads for 10 seconds in 2.0% paraformaldehyde and 2.5% glutaraldehyde in Sorensen's buffer at pH 7.25. Standard carbodiimide crosslinking was accomplished by soaking the thread for 8 hours in 50 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Pierce, St. Louis, Missouri) in 90% acetone, then rinsing exhaustively with water. Before implantation, the threads were depyrogenated [C.S. Genovesi, *In: F.J. Carleton and J.P. Agalloco (ed.), Validation of aseptic pharmaceutical processes*, p. 381, Marcel Dekker, New York, NY (1986); C.P. Prior, *In: C.S. Ho and D.I.C. Wang (ed.), Animal Cell Bioreactors*, p.460, Butterworth-Heinemann, Stoneham, MA (1991)] by soaking in 0.1 M NaOH overnight at 4°C, and sterilized by room temperature ethylene oxide (EtO) treatment.

Intermolecular crosslinkages can also be established through a dehydrothermal process (heat and vacuum) which is well-known in the art. This procedure can be performed as an additional step after crosslinking for added strength.

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Constructs may be formed from the collagen threads of the present invention by techniques for processing fibers known to those skilled in the art, e.g., knitting and weaving. Most fiber handling techniques for both natural fibers, e.g., cotton, silk, etc., and synthetic fibers, e.g., nylon, cellulose acetate, etc., should be useful in processing threads provided herein, including techniques used to produce three-dimensional textiles. See, e.g., Mohamed, *American Scientist*, 78 (1990) 530-541.

Collagen threads provided by the present invention have been used to form braided constructs (See Example 3 below), plied into yarn (See Example 4 below), and knitted (See Example 5 below). The collagen threads can also be woven using techniques known to the skilled artisan to produce woven constructs. In such applications, the numbers of collagen threads used, as well as the combination of crosslinked ("XL") and uncrosslinked threads, can be varied as desired.

A knitted tube comprising two ply yarn, a twist of one XL and one non-XL collagen thread of the present invention, has been used in the preparation of a blood vessel construct as described in Example 6 below.

The threads are then arranged into a desired shape such as a bundle, braid, or woven fabric. A multi-filament bundle can be formed by, for example, winding the threads around a fixture to produce a closed-end loop. A bundle can be used to form a multi-filamented braid of three or more bundles of threads, a two or more bundle helix, or a single twisted bundle or untwisted bundle. Loops may also be formed at the end for fastening the device.

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Collagen braids can be produced using successive braiding operations, each building upon the material previously made. For example, a braid made of 576 piles can be constructed from a 4-ply braid of collagen mono-filaments. This 4-ply braid may be produced on a 4-carrier braiding machine (NE. Buttcol, Pawtucket, Rhode Island). This braid can then be 4-ply braided two more times to produce a 64-ply construct, which can then be 3-ply braided.

The present invention also provides constructs which comprise collagen threads knitted to form a fabric. Knitting collagen threads, bundles or braids formed as described above provides a versatile technique to produce strong, porous structures. Four fabric patterns, depicting a range of porosity, are shown in Figures 2A-D. These fabrics illustrate only a few of the possible knittable structures. Collagen fabrics can be tailor-made to suit a wide variety of applications with varied porosity, elongation, strength and bulk requirements. Braids (Fig. 5) can also be made small or large, with different densities of braid angles, potentially useful for soft tissue augmentation, reconstructive and repair applications, as well as for load-bearing orthopedic prosthesis. Devices made of collagen fabrics can be produced by both flat and tubular weft knitting, and both Raschel and Tricot warp knitting. Knitting designs including, for example, open elastic meshes, dense stable fabrics, tubular structures, can be produced using starting material from individual or multiple monofilament threads to twisted or braided yarns.

One important advantage of knitting over weaving in the production of collagen fabrics is that knitting introduces closed loops at the yarn

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crossover points, allowing the material to hold sutures with very little bite, and without needing to fold the material at the suture line. In contrast, weaving merely interposes parallel yarns, resulting in a fabric more subject to fraying when cut. In addition, knitting offers more options for varying the physical character and bulk of the fabric than does weaving.

There are two basic types of knitting machines. Weft knitting machines use only a single end of yarn and individual needles cast off stitches sequentially. Warp knitting machines use many ends of yarn parallel-wound on a cylinder (the warp), and many needles (a "needle bar") cast off stitches simultaneously to produce the fabric. Because no warp is needed for weft knitting, it can be extremely valuable for evaluating experimental yarns. Ten meters of yarn (either monofilament thread, or a twisted or braided multifilament) would more than suffice for test-knitting a collagen fabric on a circular weft knitting machine, compared with over 2000 m of yarn required for test knitting on a warp knitting machine, even on a small "sample" machine.

However, although warp knitting is more demanding and requires more material, it offers distinct advantages. Because weft knitted fabrics are formed from a single end, they can unravel if that end is pulled, or if the fabric is cut in the middle and the free end is pulled. In contrast, most warp knitted fabrics do not unravel when cut. Moreover, by simultaneously using additional warps and additional needle bars, complex fabrics can be designed in which more than one "layer" of fabric is knitted at the same time. For example, the fabric used in the rat hernia repair model below,

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was knitted on three bars, for low porosity, high bulk, good stability, suture retention, and tensile strength.

There are two main categories of warp knitting machines. The Raschel type has a latched needle to hold the yarn; the Tricot type holds the yarn with a flexible bent tip, termed a "beard." Raschel machines offer a more versatile array of knitting patterns, but Tricot machines exert less stress on the fibers. Both flat and tubular structures can be made on Raschel machines; Tricot machines are mainly used to produce flat structures.

The extensibility and porosity of the fabric can be controlled by varying the braid angle. Accordingly, the skilled artisan can tailor the properties of the fabric to the physical properties of the area to be treated. For example, repair of a hernia would require a fabric with low extensibility, i.e., a stiff fabric. Whereas, it may be preferable to use a fabric having high extensibility when treating a wound such as a deep ulcer.

Once fabricated, the device can be used to replace or enhance the function of a ligament, tendon to other structure that has been damaged. The present invention is illustrated by replacement of the ACL ligament of the knee and by hernia repair. However, the constructs and methods of the present invention are suitable for use in repair or replacement of ligaments, tendons and other structures generally. The fabric can be used in known surgical methods, for example, in methods of hernia repair, blood vessel replacement, prolapse support and chest wall reconstruction after

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trauma or tumor resection [Lampi, *Thorac. Cardiovac. Surgeon*, 36:157-158 (1988)].

The following non-limiting Examples describe methods of fabrication and *in vitro* and *in vivo* testing of the device of the present invention.

The invention will be further understood with respect to the following examples which are purely exemplary in nature and are not meant to be utilized to limit the scope of the invention.

An apparatus similar to that shown in Fig. 1 was used in carrying out the work described in the following examples.

The baths described below were used in the following examples unless otherwise noted:

Dehydrating Bath

1200g PEG (8000), 20g of monobasic sodium phosphate (monohydrate) and 71.6g of dibasic sodium phosphate (anhydrous) were dissolved approximately 4000 ml water in the 10 L vessel and mixed well until the solids were dissolved. The pH was then adjusted to $7.50 \pm .05$ with 1N NaOH and water added to a final volume of 6000 ml.

Rinse Bath

Phosphate Buffered Saline (PBS) was prepared by dissolving 0.35g Potassium phosphate monobasic, 7.5g Sodium phosphate dibasic

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heptahydrate, and 22.5g Sodium chloride in water and adjusting the final volume to 5000 ml.

EXAMPLE 1 - COLLAGEN THREAD PRODUCTION

Materials and Equipment

1. Collagen: Collagen was prepared as disclosed in U.S. Serial No. 07/407,465, *supra*, and stored at 4°C until ready to use. Collagen in 0.05% acetic acid at 5.0 mg/ml was degassed by centrifugation prior to use.
2. Beckton Dickinson 60 cc syringe with widely spaced O-rings.
3. Popper & Sons, Inc. blunt stainless steel needle, 18 gauge, with silicone leader tubing and bridge.
4. Harvard Apparatus Syringe Pump (Pump 22).
5. An 18 foot long PVC dehydration trough 2 inches in diameter, with Masterflex Pump and norprene tubing.
6. Dehydrating Agent 20: PEG (8000 M.Wt.) from Spectrum, phosphate-buffered at approximately pH 7.50.
7. A rinsing trough, 6 feet in length.
8. Rinsing bath (1/2 x PBS).

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9. Drying cabinet with pulleys and heated blowers (2).
10. Level wind uptake spool and driver.

Extrusion

Approximately 6000 ml of dehydrating agent was poured into the dehydrating trough and the recirculating pump was started. The dehydration agent velocity was about 5 cm/sec for collagen solutions having viscosities of about 400 cs^{-1} .

Approximately 4000 ml of the rinsing bath (1/2 x PBS) was added to the rinsing trough.

A needle was placed into the dehydrating agent approximately 12 inches from the upstream end. The collagen syringe barrel was attached to the syringe pump, the pump set to at an infusion rate of about 2.5 ml/min., and the infusion pump started.

When enough slack was generated in the dehydration trough, the thread was manually transferred through the rinsing trough and disposed over the first pulley in the drying cabinet. The thread typically sat for about 3 minutes in the rinsing trough. The heated blower was then turned on set to "low", i.e., about 30-45°C. Gradually, as the thread dried, the collagen thread was then carefully disposed over the pulleys in a zig-zag fashion.

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The free end of the thread was wound onto the uptake spool. The speed of the uptake spool was set so that the thread emerged dry to the touch from the cabinet.

Continuous thread of up to 75 meters has been produced.

EXAMPLE 2 - CROSSLINKING COLLAGEN THREAD

The collagen thread was drawn through a 6 inch polycarbonate trough containing 2.0% paraformaldehyde, 2.5% glutaraldehyde, and sorensen's phosphate buffer, (Electron Microscopy Science), pH 7.4. The thread was then rinsed in a 6 inch trough containing 0.5X sorensen's phosphate buffer. The dwell time in each bath was 5 seconds. The thread was then air dried for 15 seconds and collected onto a stainless steel shaft.

EXAMPLE 3 - BRAIDING COLLAGEN THREAD

A harness braiding machine (New England Butt Co., Providence, RI, USA) was used to braid collagen thread, both XL and non-XL, prepared in accordance with Examples 1 and 2, above.

The number of spools to be braided was varied, typically from 3 to 8. The type of thread was also varied as well (crosslinked, noncrosslinked, etc.). Care was taken to spool the thread onto a suitable carrier such that the thread unspooled freely and without snagging or excessive tension.

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Braid tightness was varied by varying the gear ratio according to the instructions provided with the machine. Typically, the collagen thread to be braided was wound onto cylindrical stainless steel spools, approximately 0.8 inches in diameter. These spools were mounted onto the braiding carousel, and the thread gathered according to the instructions provided with the machine. In one run, a braid was composed of four strands: two threads of uncrosslinked (non-XL) collagen, and two threads of collagen which had been crosslinked (XL) in glutaraldehyde (supra). Equal lengths of thread were used on each spool to be braided. The load at break was greater than the sum of the load at break of the constituent threads.

EXAMPLE 4 - PRODUCTION OF COLLAGEN YARN

Collagen thread produced in accordance with Examples 1 and 2 above has been plied into yarn, as other types of thread would be (e.g., cotton, polyester, etc.). Twisting methods and machinery used in producing this yarn are standard for the textile industry. Persons with ordinary skill in producing yarns from other types of thread may apply those same methods using the collagen thread of the present invention to achieve the desired result.

In one standard run, one uncrosslinked collagen thread and one crosslinked thread were each given a Z-twist at 6 twists per inch (tpi); then the two were twisted together in an S-twist with 4 tpi.

Yarns may be made of any desired number of collagen threads, produced per Examples 1 and 2 above, in any combination of crosslinked

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and uncrosslinked. For example, 60 ply yarn has been made from uncrosslinked collagen threads of the present invention. In producing this 60 ply yarn, first 4 collagen threads were twisted together; then 3 of the resultant 4-ply strands were twisted together in the opposite direction; and finally 5 of the resultant 12-ply strands were twisted in the opposite direction.

EXAMPLE 5 - KNITTING COLLAGEN THREAD AND YARN

Collagen threads and/or braided or plied yarns produced in accordance with Examples 1-4 above, have been knitted into tubular or flat fabrics and meshes. The knitting methods and machinery used in the production of these articles are standard for the textile industry. Persons with ordinary skill in producing tubular or flat fabrics or meshes from other types of thread and/or yarn may apply those same methods using collagen thread and/or yarn to achieve the desired result.

Both a circular knitter: Lamb, Inc. (Agawam, MA, USA) and a Warp Knitter: Karl Mayer Maschinen Fabrik (Germany) have been used to produce knitted articles. In one run, a tube of 3 mm inner diameter was knitted on the warp knitter from 2 ply yarn (1 non-XL and 1-XL collagen thread) prepared in accordance with Example 4 above with a double needle for using four ends and 1/0-1/2 (closed stitch).

A wide range of diameters is possible, e.g., from about 4 mm to about 10 cm.

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A circular knitter (Lamb, Inc., Agawam, MA, USA) was used in a warp knitting process wherein:

Many ends came together and knit. This process produces circular knitted tubes which will not unravel when cut. Many parameters, including tension, type of stitch, number of ends, number of knitting bars, etc. can be varied.

EXAMPLE 6 - PRODUCTION OF SUPPORT FOR BLOOD VESSEL EQUIVALENT

3 mm knitted tubes produced in accordance with Example 5 above have been used to produce collagen constructs. Such constructs were produced in accordance with Example 8 of copending U.S. Application Serial No. 07/505,678, except that the 3 mm knitted tube was used in place of the DACRON® mesh.

It is understood that the examples and embodiments are for illustrative purposes only, and that various modifications or changes in light thereof that will be suggested to persons skilled in the art, are to be included in the spirit and purview of this application and the scope of the approved claims.

EXAMPLE 7 - COLLAGEN PRODUCTION

Type I collagen was extracted from bovine tendons as follows. Calf hooves were collected from a slaughter house and the digital extensor

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tendons were manually dissected out from surrounding fascia. The tendons were rinsed with water, ground through a ¼" grinder plate (Hobart, Dedham, Massachusetts) and washed at 4°C in three changes of 90 volumes (v/w) of 3.7 mM sodium phosphate dibasic, 0.35 mM potassium phosphate monobasic, and 51 mM NaCl at pH 7.50. Appropriate mixing was achieved using a 15 L suspension vessel (Bellco, Vineland, New Jersey) at approximately 100 rpm. Each of the three salt solution changes were mixed with the ground tendon for 2 hours, then pumped out and discarded. Acid extractable collagen was solubilized from the solid material by mixing as described above in 90 volumes (v/w) of 0.5 M acetic acid for 72 hours at 4°C. The solubilized collagen was separated from the insoluble residue by centrifugation at 26,000g for 30 min. at 4°C. The solution was filtered under positive pressure through a 5 µm nylon screen, and Type I collagen was purified by repeated precipitation in 0.9 M NaCl and redissolution in 1 M acetic acid.

The collagen solution was dialyzed at 4°C in 2.5 cm dialysis tubing, MW cutoff 8000 (Baxter, Bedford, Massachusetts), against 3 changes of 20 volumes (v/v) of 8.8 mM acetic acid. The solution was filtered at 4°C through a 6 µm filter unit (Pall, Fajardo, Puerto Rico) and concentrated at 4°C to 5.0 mg/ml in a hollow fiber ultrafilter (A/G Technology, Needham, Massachusetts) with a 100,000 MW cutoff. The collagen solution was then degassed by centrifugation at 14,000g for 30 minutes at 4°C.

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EXAMPLE 8 - THREADMAKING

The threadmaking process used in this Example is shown schematically in Figure 1. A 140 ml syringe containing a 5.0 mg/ml collagen solution in 8.8 mM acetic acid was loaded in a syringe pump (Harvard Apparatus, South Natick, Massachusetts) set to infuse at 2.50 ml/min. Silicone tubing (1/8-inch I.D.) connected the syringe to an 18-gauge blunt stainless steel needle immersed in one end of an 18-foot long, 2-inch diameter PVC trough containing 5 L of 20% polyethylene glycol (PEG), MW 8000 (Spectrum Chemicals, New Brunswick, New Jersey), in 94 mM sodium phosphate dibasic and 24 mM sodium phosphate monobasic at pH 7.55. A peristaltic pump was used to recirculate the PEG solution such that the fluid velocity at the needle tip was about 4 cm/sec. The circulating PEG solution drew the collagen away from the needle orifice, thus forming a continuous filament. The collagen gelled on contact with the neutral pH solution and the nascent thread began to dehydrate due to the osmotic pressure gradient formed between the collagen and the PEG solution. The coagulation trough was configured such that the residence time of the thread in the bath was approximately 4 minutes. As the thread accumulated, it was manually transferred to a 6-foot long trough filled with 5.5 mM sodium phosphate dibasic, 0.5 mM potassium phosphate monobasic and 75 mM NaCl at pH 7.10, and remained there for 5 to 10 minutes.

The thread was then partially dehydrated by passing it through a 2-foot long trough containing 70% isopropanol, and dried under tension by drawing it over a series of Teflon pulleys inside a cabinet heated with air

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blowers. To prevent the collagen from being heat denatured, the thread was kept in motion, and was always at least 15 cm from the blowers. Finally, the thread was spooled onto a level winding device. The tension on the thread during the drying was such that its length doubled before it emerged dry from the cabinet. The total residence time in the cabinet was approximately 3 minutes.

Once the filament was threaded through the entire system, it could be produced continuously; the operator needed only to keep the coagulation and rinsing reservoirs filled with enough slack to permit reeling. In general, a yield of about 1.2 m of thread per ml of collagen solution was obtained. Production rates of this laboratory scale apparatus were consistently over 100 m/h.

Histology

Light microscopy specimens were fixed in 10% buffered formalin, dehydrated in a graded ethanol series, infiltrated with Tissue-Prep 2 paraffin (Fisher Scientific, Fair Lawn, New Jersey) and embedded in Paraplast Xtra (Sherwood Medical, St. Louis, Minnesota). Five μm sections were stained with hematoxylin and eosin. TEM specimens were prepared [H.B. Lin, *Biomaterials*, 13:905-914 (1992)] by fixing for 48 hours in 2.0% paraformaldehyde, 2.5% glutaraldehyde, and 1% acrolein in 0.1 M sodium cacodylate at pH 7.50, post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate, and stained *en bloc* with 2% uranyl acetate. After secondary fixation, all specimens were dehydrated in a graded ethanol series and propylene oxide, and embedded in Epox-812 (Ernest F. Fullam, Rochester, New York). Ultrathin sections (about 700 nm) were stained with uranyl

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acetate and lead citrate, and examine using a Jeol JEM100S transmission electron microscope at 80 kV. SEM specimens were fixed as for TEM, dehydrated in a graded ethanol series, and critical point dried using a Tousimis Autosamdri-814. Samples were mounted and coated with 60/40 gold/palladium and examined using an ISI DS-30 scanning electron microscope.

Figure 3a shows a typical micrograph of a collagen threads. The outer surface is smooth, and the width consistent throughout the field. The same field viewed in plane polarized light (Figure 3b) shows a strong birefringence pattern, due to the orientation of collagen fibrils in line with the thread axis. Scanning electron microscopy (Figure 4a) reveals that the threads are not round in cross section, but rather flattened. This flattening is due to contacting the wet thread on the pulleys within the drying cabinet. Collagen threads dried by suspending them in air have more circular cross-sections. Transmission electron microscopy (Figure 4b) shows an interior of filamentous elements about 10 nm in diameter, encased by a layer of larger fibrils showing the characteristic 67 nm banding pattern.

Thread Testing

Thread strength was determined by mounting 50 mm sample lengths in a force gauge (Chatillon Corp., Agawam, Massachusetts) and pulling at 50% strain per minute until failure. Ultimate elongation and load at break were noted. Thread size was measured two ways. Diameter was measured microscopically (10X) using a measuring eyepiece, averaging the readings taken on at least five thread samples in at least five random

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locations. More characteristics of textile fibers, thread mass per length, or denier (mass in grams per 9000 meters of length) was also measured. Typically about 80, the denier can be varied from about 15 to about 300 by altering the collagen infusion rate, the coagulation bath flow rate and/or needle (orifice) size.

Shrinkage temperature, a measure of the stability of the collagen triple helix was measured by immersing a 5 to 7 cm loop of thread loaded with 2.5 g in 1.0 mM potassium phosphate monobasic, 11 mM sodium phosphate dibasic, and 150 mM NaCl at pH 7.30, and heating at 1°C per minute until shrinkage occurred. The temperature at which the sample shrank by at 10% was recorded as the shrinkage temperature.

Table II set out below shows mechanical strength data of collagen and other threads. The results are similar to published tensile strengths for continuous collagen threads [H.B. Lin, *supra*, (1990)]. Both EDC and glutaraldehyde crosslinked threads have wet ultimate tensile stresses (UTS) of around 25 MPa, about one-third that of fresh tendon [D. Greenwald, *Plas. Surg. Res. Council*, In press (1993)].

TABLE II

Ultimate tensile strength (UTS) of collagen threads produced by the method described (n = 10) and reconstituted collagen fibers published elsewhere [Y.P. Kato, *supra* (1990)]. (XL = crosslinked. Standard EDC and glutaraldehyde crosslinking were carried out as described in the methods).

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	UTS (MPa)
Dry Collagen Thread (non-XL)	224 \pm 19
Dry Collagen Thread (EDC XL)	197 \pm 18
Dry Collagen Thread (glutaraldehyde XL)	175 \pm 19
Wet Collagen Thread (non-XL)	1.2 \pm 0.2
Wet Collagen Thread (EDC XL)	23.9 \pm 2.7
Wet Collagen Thread (glutaraldehyde XL)	27.7 \pm 3.1
Wet Collagen Thread (non-XL) *	2.4 \pm 0.46
Wet Collagen Thread (cyanamide XL) *	17.4 \pm 2.08
Wet Collagen Thread (glutaraldehyde XL) *	44.1 \pm 6.62

* [Y.P. Kato, *supra*, (1990)]

The mechanical properties of the collagen threads depend on the degree of covalent crosslinking, but tensile strength and shrinkage temperature were found to be affected at different rates. Figure 6a depicts the timecourse of shrinkage temperature increase for EDC concentrations up to 50 mM and thread residence times up to 24 hours. Figure 6b depicts the timecourse of wet strength development for the same experimental groups. The data show that shrinkage temperature is elevated faster and reaches a maximum value sooner than wet tensile strength does for a given EDC concentration. Moreover, threads may have similar shrinkage temperatures and very different wet strengths, suggesting that differing mechanisms are responsible for each.

As shown in Figure 6, both shrinkage temperature and wet strength increased with increased crosslink time and EDC concentration, but at

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different rate: increased helix stability is achieved sooner than load-bearing capacity. This result is most likely due to crosslinks forming within a single collagen molecule (intramolecularly), sooner than between adjacent molecules (intermolecularly). Intramolecular crosslinks may stabilize the helices, but cannot increase strength, as such bonds do not provide any means of load sharing between molecules.

EXAMPLE 9 - ACL REPLACEMENT

Device Fabrication

Collagen braids of up to 576 plies were produced using successive braiding operations, each building upon the material made previously. For the 576 braid, a 4-ply braid of collagen monofilaments was first produced on a four-carrier braiding machine (N.E. Butt Co, Pawtucket, Rhode Island). This braid was then 4-ply braided two more times to produce a 64-ply construct, which, in turn, was 3-ply braided by hand twice. A 600-ply collagen bundle was produced by winding collagen thread 300 times around a 20 cm long fixture to produce a closed loop, which was then removed, saturated with water and air dried at 4°C tension.

Canine Ligament Model

In this Example, the prosthetic device was used to replace the anterior cruciate ligament (ACL) in a dog model. Two constructs, one 576-ply braid and one 600-ply bundle, were fabricated as described above, EDC crosslinked by the standard procedure, and EtO sterilized. The left stifle joints of two beagles were exposed by lateral arthrotomy. The ACL's were excised, and either the braided or bundled prosthesis was implanted using

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an over-the-top technique [Montgomery, et al., *Clin. Orthop. Rel. Res.*, 231:144-153 (1988)]. Both constructs were implanted between the periosteum and the bone on both tibial and femoral ends, and through the infrapatellar fat pad. The prosthesis were fixed to bone using two titanium staples both proximally and distally, driven using a Staplizer compressed air gun (3M, St. Paul, Minnesota) at 110 psi input pressure. After the joint capsules were closed with size 2-0 Vicryl suture, the prostheses were supported laterally with loops of size 2 Maxon sutures placed from the femorofabellar ligament to the tibial tuberosity, parallel to the prosthesis direction.

Twelve weeks after implantation, gait analysis was performed on the dogs using a force plate analyzer; the animals were then terminated. At explant, synovial fluid samples were taken for analysis. Proximal, mid-substance, and distal portions of the explant were submitted for histological processing (light and TEM) as described below. Bony specimens were demineralized in Decal 5% nitric acid (Poly Scientific, Bay Shore, New York) after fixation.

By 12 weeks, the dogs were nearly normal in gait; there was some disuse atrophy of the muscles in the left hind limb, which was returning to normal during the last few weeks of the experiment. Force plate analysis showed the operated limb at about 70% use of the unoperated limb, and a 20% increase in force applied to the right forelimb as lameness compensation.

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The joints appeared stable. Collagen threads were readily apparent only at the stable attachment sites. Within the joints, in the position and orientation of the removed ligament, collagenous "neoligaments" were seen, well adhered to bone at both ends. Synovial fluid from both the control and operated joints tested within normal parameters for color, turbidity, specific gravity, protein content, and cellularity.

Light microscopy in the three regions examined showed different remodeling modes. Adjacent to the staple attachment sites (Figure 8a), the collagen threads remained largely intact and surrounded by a disorganized cellular ingrowth. The explant mid-substance, although slightly hypercellular (Figure 7b) contained large collagen bundles parallel to the long axis of the ligament. At the tibial junction within the joint capsule (Figure 6c), the neoligament is seen interdigitating with cartilaginous prominences, reestablishing natural fixation.

TEM photographs (Figure 8a) of the neoligament and mid-substance show connective tissue that is not as well organized as with the contralateral control (Figure 8b). However, numerous normal size collagen fibrils are evident, organized parallel to the long axis. Fibril diameter measurements in the explant material show a distribution pattern similar to that found in the control (Figure 9).

In this animal model, the sequence of events after device implantation followed those typically found as part of a normal wound healing response. An initial, transient inflammatory response, generally resolved by 12 weeks, was followed by cellular ingrowth, revascularization,

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and the generation of new connective tissue. The implanted devices continued to perform their required function during the remodeling period.

The actual time course of remodeling is likely to be dependent upon 4 factors: implant, site, degree and type of crosslink [Y.P. Kato, *supra* (1990)], implant size and density, and the mechanical forces acting on the implant. The ligament implant was seen to respond to its immediate stress environment (Wolff's law [Y.C. Fung, *Biomechanics*, Springer-Verlag, New York (1981)]): the collagen threads at the staple were not load bearing and were hardly remodeled at 12 weeks. The remainder of the implant, which was under load, remodeled to a much greater degree, and differently in the two locations observed.

EXAMPLE 10 - HERNIA REPAIR FABRIC

This fabric was manufactured first by producing a 2-ply collagen yarn. Two monofilament strands of DFC thread were each twisted at 1.5 twists-per-inch (tpi) in the Z-direction; then, they were twisted together at 2.5 tpi in the S-direction. The result was a 2-ply yarn which does not unravel or spring. This yarn was then warped using a conventional single end warper (more convenient for trial quantities than a creel) onto an ordinary knitting beam. The hernia repair fabric under investigation used three such beams on a 20-gauge Tricot sample knitting machine in a pattern designed for high bulk and low extensibility. The stitch design is as follows:

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Front bar (#1):	0-1/1-0//
Middle bar (#2):	1-0/4-5//
Back bar (#3)	4-5/1-0//

This fabric was evaluated as an abdominal wall replacement in the rat model, using a full muscle layer defect measuring 2 cm by 2 cm. Before implantation, the fabric was cleaned with acetone, crosslinked with 50 mM EDC in 90% acetone at room temperature overnight, depyrogenated in 0.1 N NaOH at 4°C overnight, and cold chemical sterilized. The fabric could also be sterilized (dry) by gamma irradiation or ethylene oxide.

RAT ABDOMINAL REPAIR MODEL

In this example, the collagen fabric was examined for its ability to close a full-thickness abdominal excision in a rat model. A 2 cm x 2 cm full-thickness abdominal wall defect was created in each of 5 Sprague-Dawley rats. A 2.5 cm x 2.5 cm piece of collagen fabric (Fig. 2D) was sutured over the defect using six 4-0 polypropylene, with a 0.25 cm overlap around the perimeter. Additional continuous sutures were placed around the fabric perimeter, through the fabric and muscle. At timepoints of 3 weeks and 12 weeks, the animals were examined for herniation and mechanical stability of the implant. The implants, along with a margin of surrounding tissue, were then removed and fixed for histological processing as described below. The area of the repair was assessed by tracing the perimeter of the wound.

All animals were healthy for the duration of the experiment. No abdominal herniation was observed up to 12 weeks postimplantation. On visual inspection at 3 weeks, the fabric was a dark-pink color, suggesting good neovascularization. The fabric was surrounded and embedded with. Blood supply to the tissue within the fabric was provided from a single

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small projection of the omen-turn (about 2 mm wide) to the underside of the fabric. No visceral adhesions were noted. Histology at 3 weeks (Fig. 10) showed a vigorous cellular infiltrate, with numerous fibroblasts and some macrophages. Abundant matrix deposition could be seen in the interstices of the fabric.

EXAMPLE 11 - BLOOD VESSEL REPLACEMENT

This mesh-like fabric was manufactured first by producing a 2-ply collagen yarn, and creating a single end warp as described above. Four such warps were used on a Raschel Double Needle bar knitting machine, to create a tubular structure in a pattern designed for low distensibility and optimum porosity. The stitch design is described as follows:

Side A, front bar:	1-0/2-3//
Side A, back bar:	2-3/1-0//
Side B, front bar:	1-0/2-3//
Side B, back bar:	2-3/1-0//

This fabric was crosslinked as described above, and incorporated in a vascular prosthesis as disclosed in U.S. Patent Application Serial No. 08/505,678. The prosthesis was then implanted in a dog model.

This invention has been described in detailed reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

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WHAT IS CLAIMED IS:

1. A prosthetic device comprising an arrangement of collagen threads, each thread formed from a solution of collagen molecules, wherein the arrangement provides a scaffold for the infiltration and population of host connective tissue cells.
2. The device of claim 1, wherein said threads are crosslinked.
3. The device of claim 2, wherein said crosslinks are formed by a chemical crosslinking reagent.
4. The device of claim 3, wherein said crosslinking reagent is selected from aldehydes and carbodiimides.
5. The device of claim 1, wherein said threads have an ultimate tensile greater than about 1.0 MPa.
6. The device of claim 2, wherein said crosslinked collagen threads have an ultimate tensile strength of about 45 MPa.
7. The device of claim 1, wherein said threads have an ultimate tensile strength from about 1.0 MPa to about 100 MPa.
8. The prosthetic device of claim 1, wherein the arrangement comprises a bundle, braid or knitted fabric.
9. A prosthetic ligament comprising an arrangement of collagen threads, each thread formed from a solution of collagen molecules, wherein the arrangement provides a scaffold for the infiltration and population of host connective tissue cells.

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10. The ligament of claim 9, wherein said ligament is a prosthetic anterior cruciate ligament.
11. A prosthetic ligament of claim 9, wherein the arrangement comprises a bundle or braid.
12. The prosthetic ligament of claim 9, wherein said bundle is formed from a continuous collagen thread.
13. The prosthetic ligament of claim 9, wherein said threads comprise a mixture of crosslinked and non-crosslinked threads.
14. The prosthetic ligament of claim 12, wherein said non-crosslinked threads are positioned on the outside of the bundle.
15. The prosthetic ligament of claim 10, further comprising a closed loop at least one end.
16. A prosthetic device comprising a knitted fabric formed from collagen threads, each thread formed from a solution of collagen molecules.
17. The prosthetic device of claim 16, wherein the fabric is warp knitted.
18. The prosthetic device of claim 8, wherein said knitted fabric has a predetermined extensibility and porosity.
19. A method for controlling the extensibility or porosity of a knitted fabric formed from collagen threads, each thread formed from

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a solution of collagen molecules comprising varying the knit angle.

20. A method of regenerating connective tissue *in vivo* comprising the steps of:
 - (a) providing a prosthetic device in accordance with claim 1; and
 - (b) implanting said prosthetic device in the vicinity of the connective tissue to be regenerated.
21. A method of regenerating ligamentous tissue *in vivo* comprising the steps of:
 - (a) providing a prosthetic ligament of claim 9; and
 - (b) implanting said prosthetic ligament into a joint by surgical procedures.
22. A woven fabric formed from collagen threads, wherein each thread is formed from a solution of collagen molecules.
23. A method for treating a wound comprising the steps of:
 - (a) providing a prosthetic device of claim 16; and
 - (b) contacting said device with said wound.

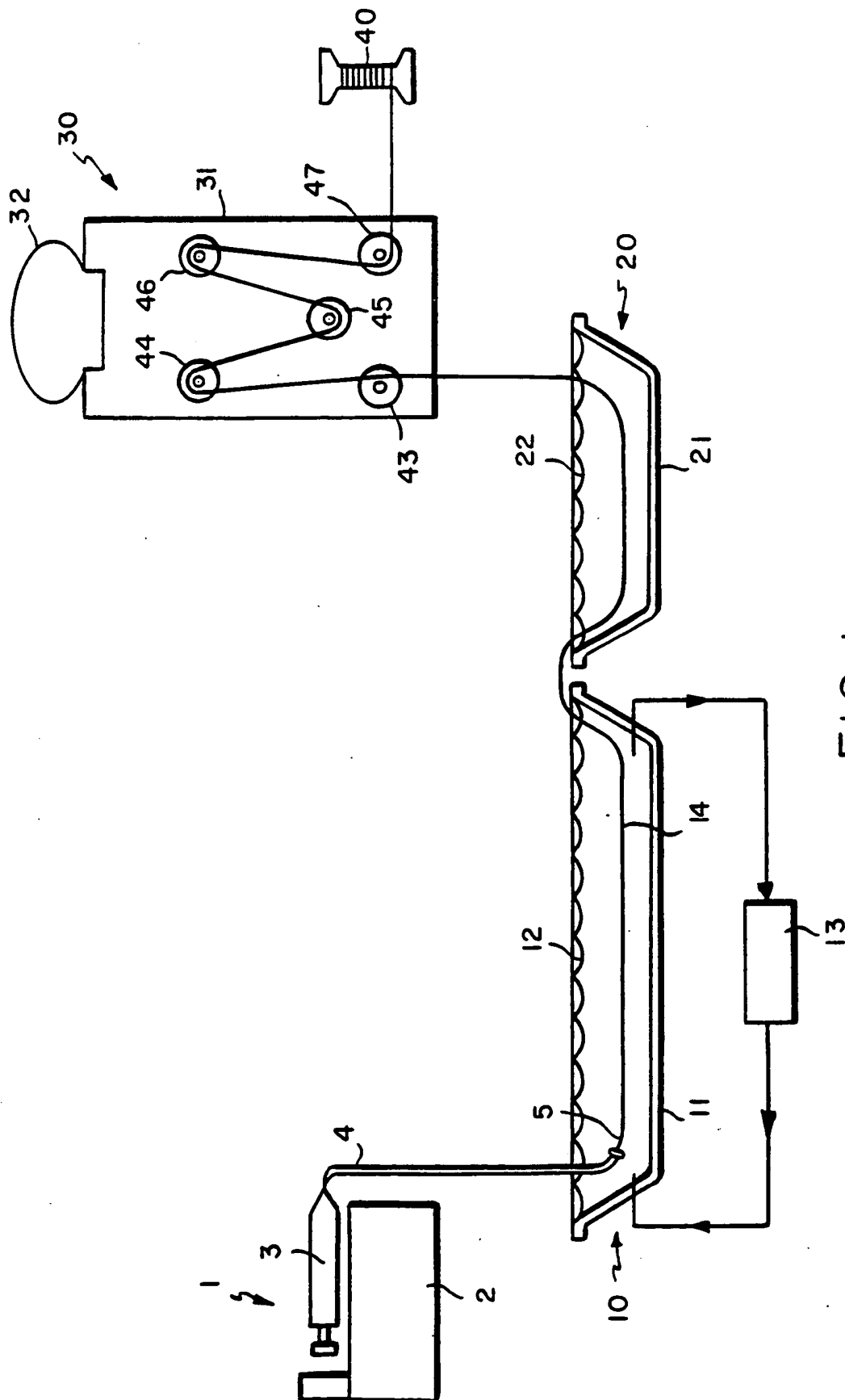


FIG. 1

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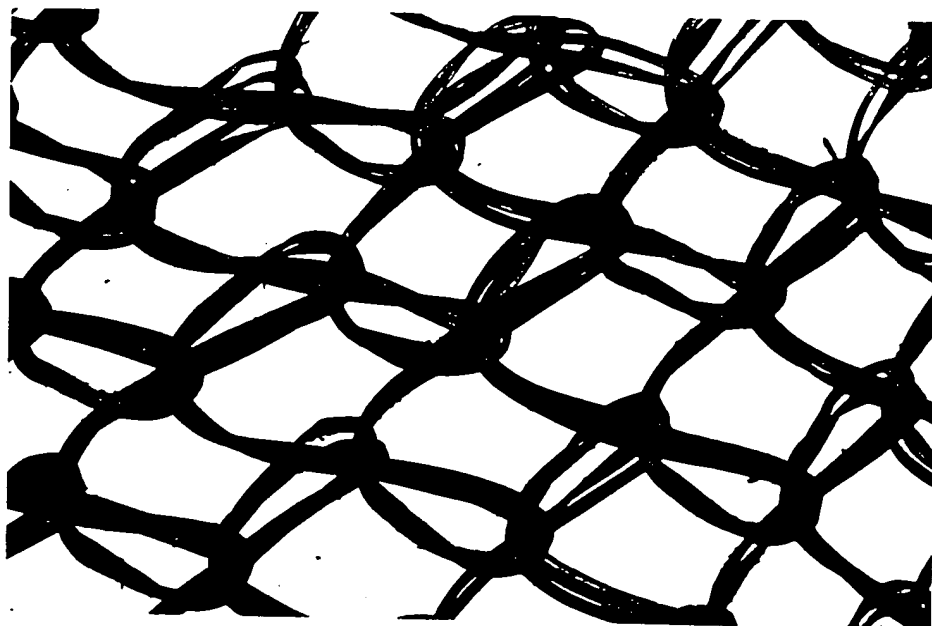


FIG. 2A

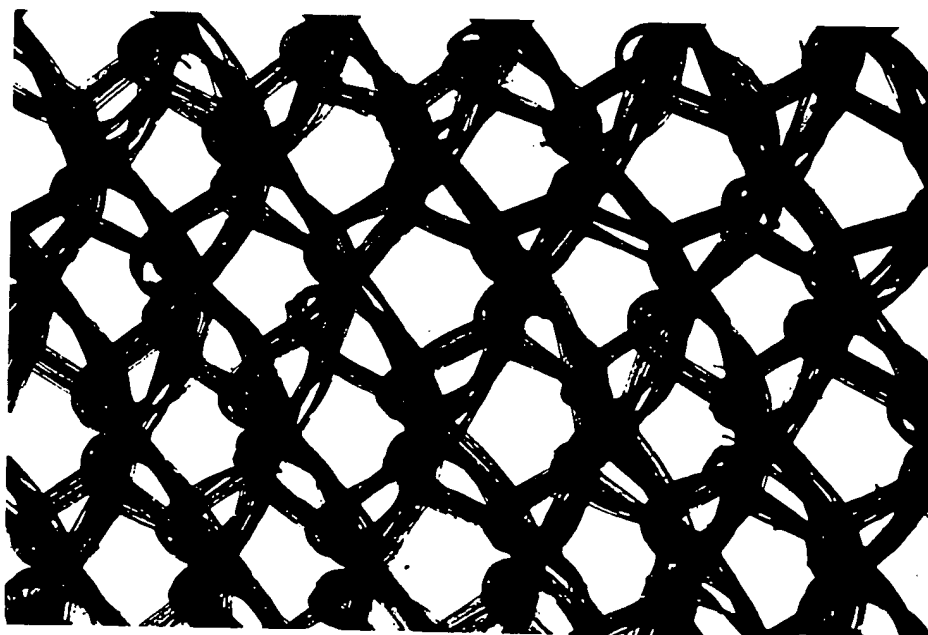


FIG. 2B

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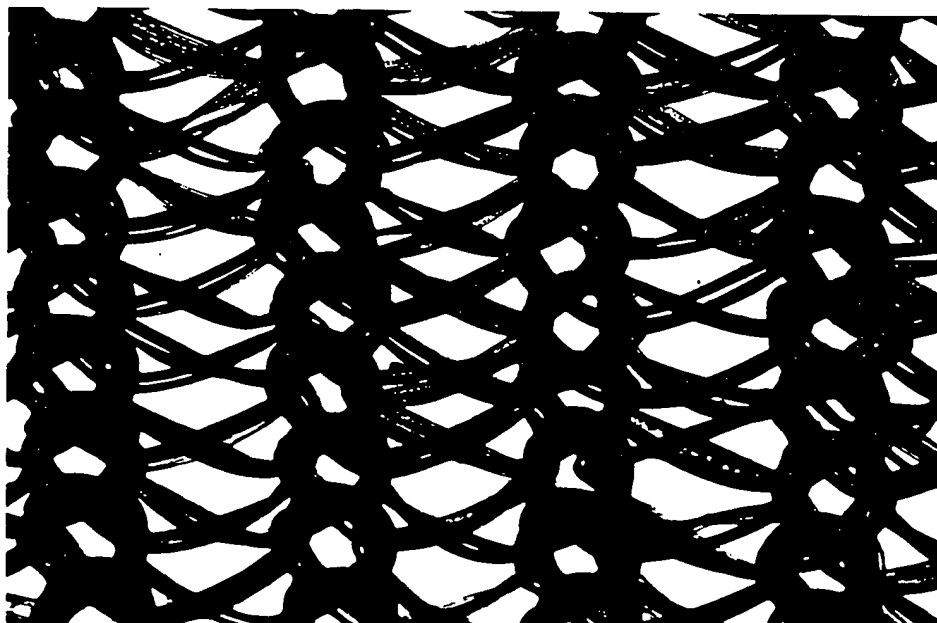


FIG. 2C



FIG. 2D

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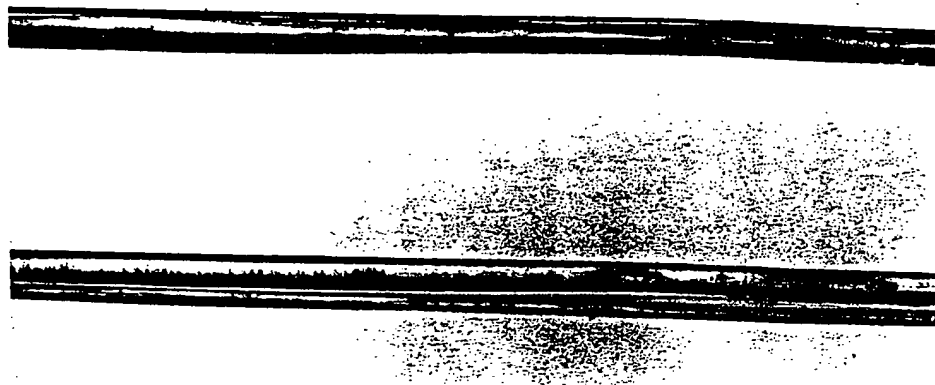


FIG. 3A

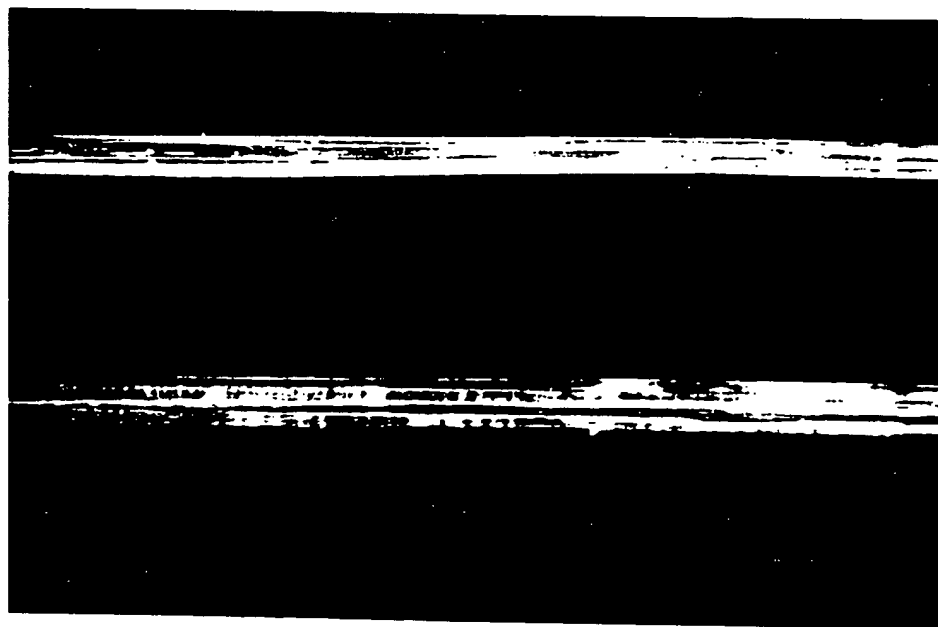


FIG. 3B

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FIG. 4A



FIG. 4B

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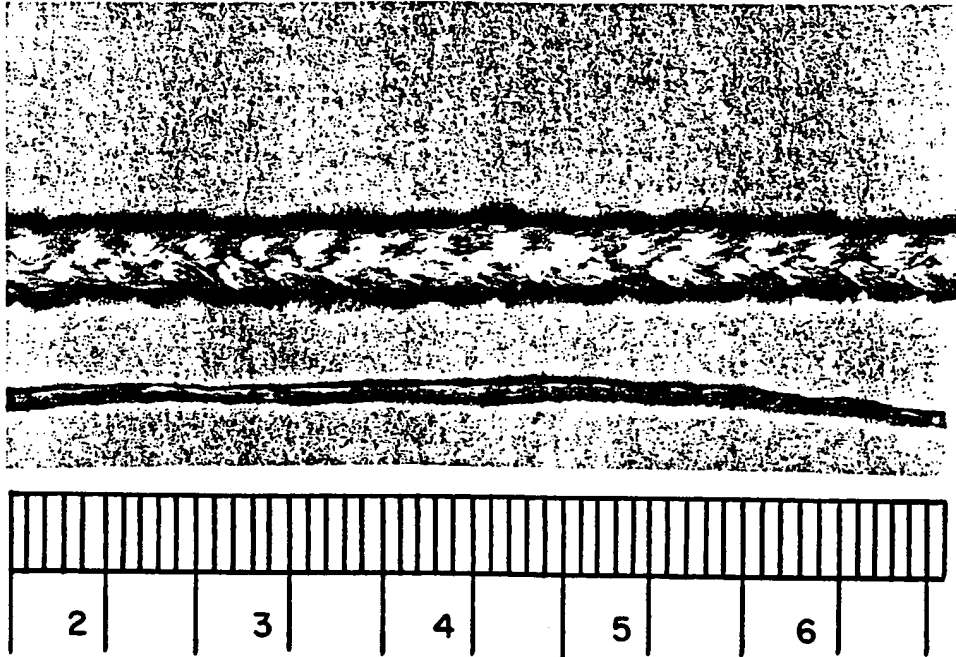


FIG. 5A

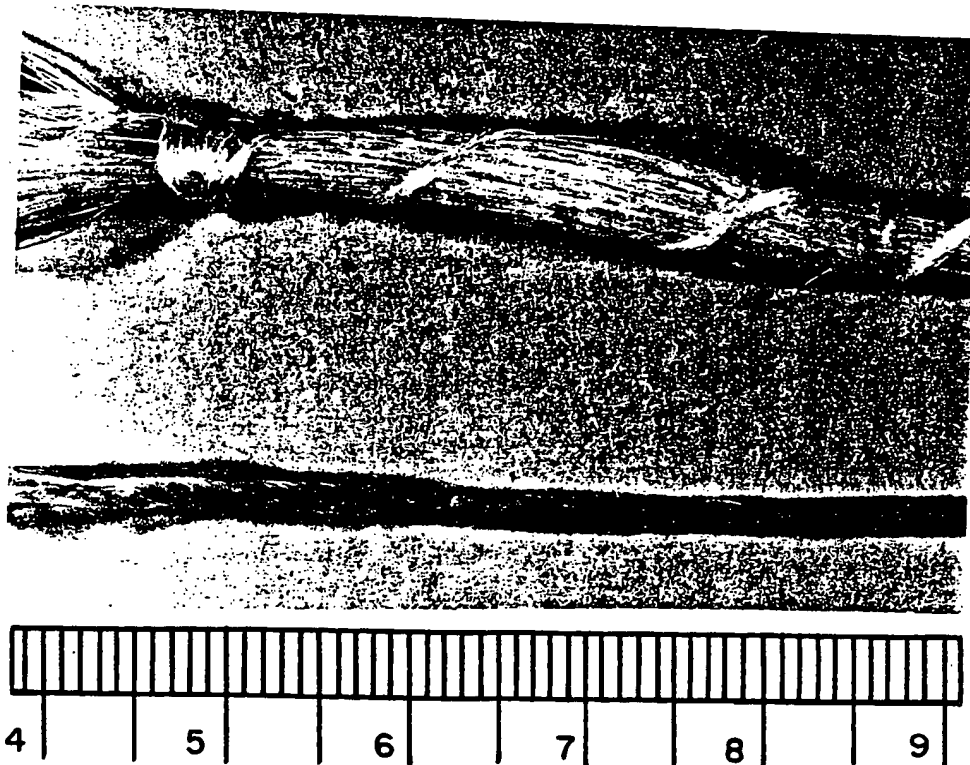


FIG. 5B

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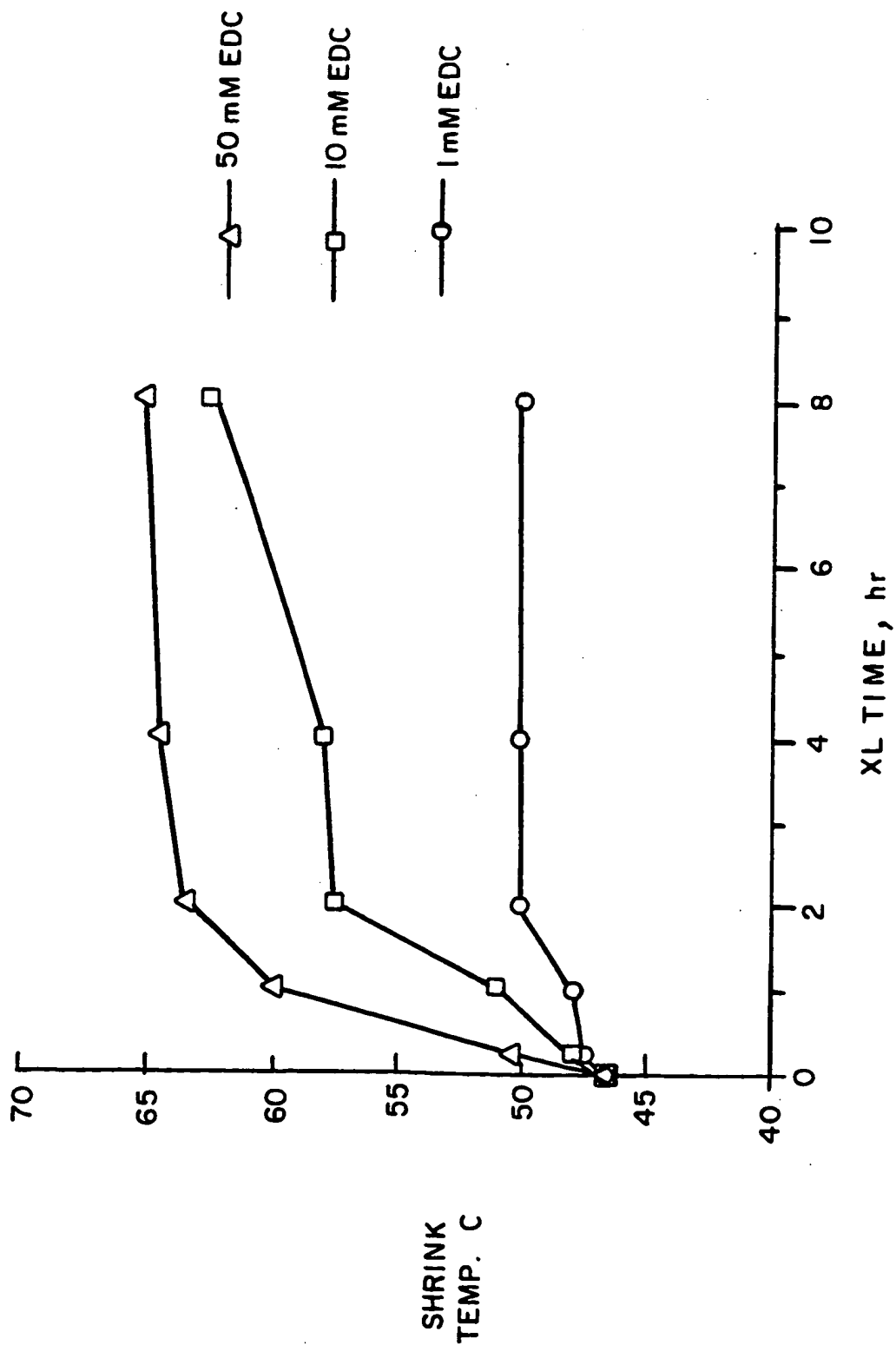


FIG. 6A

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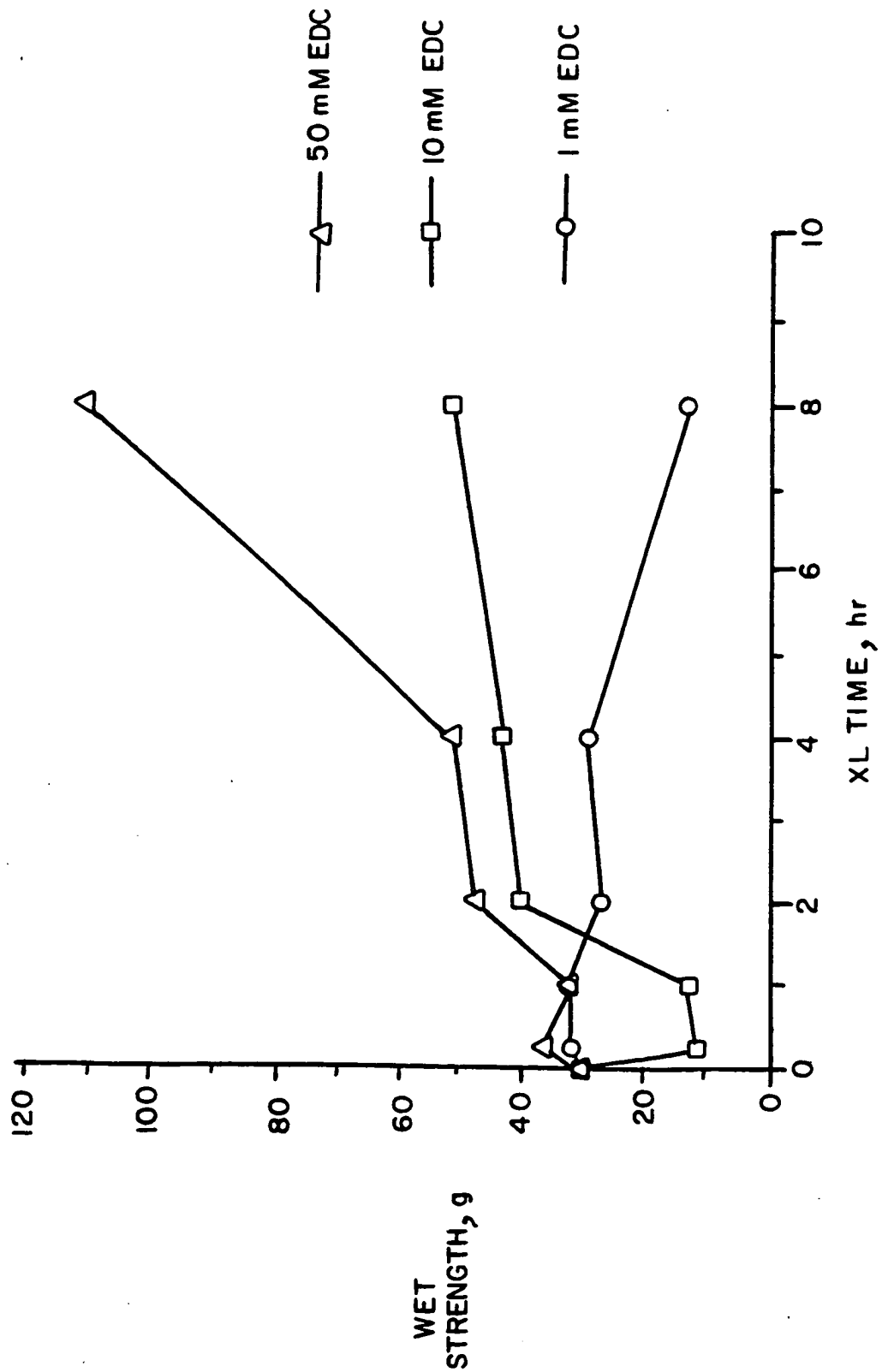


FIG.6B

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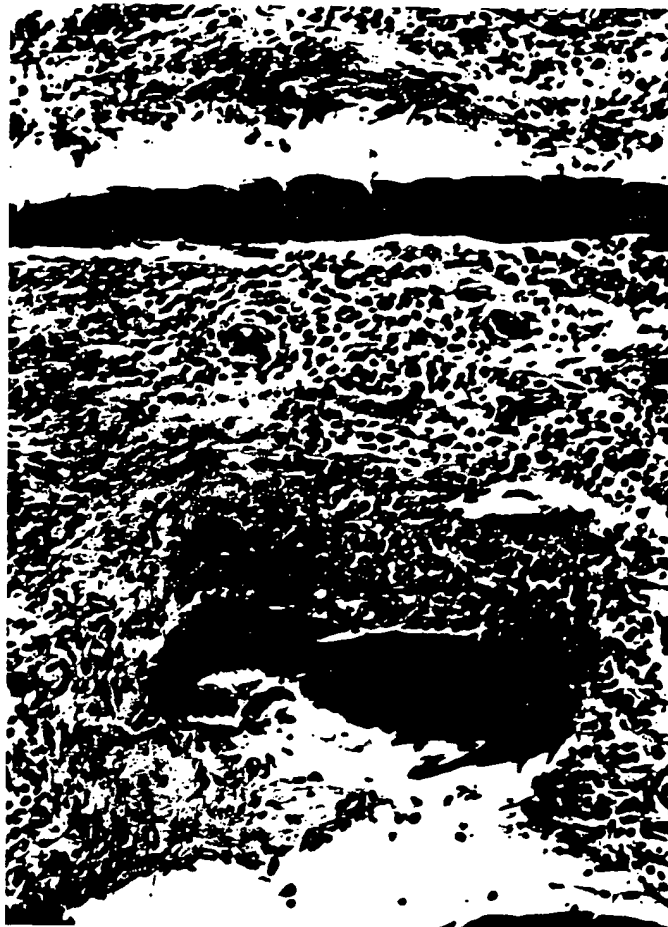


FIG.7A



FIG.7B

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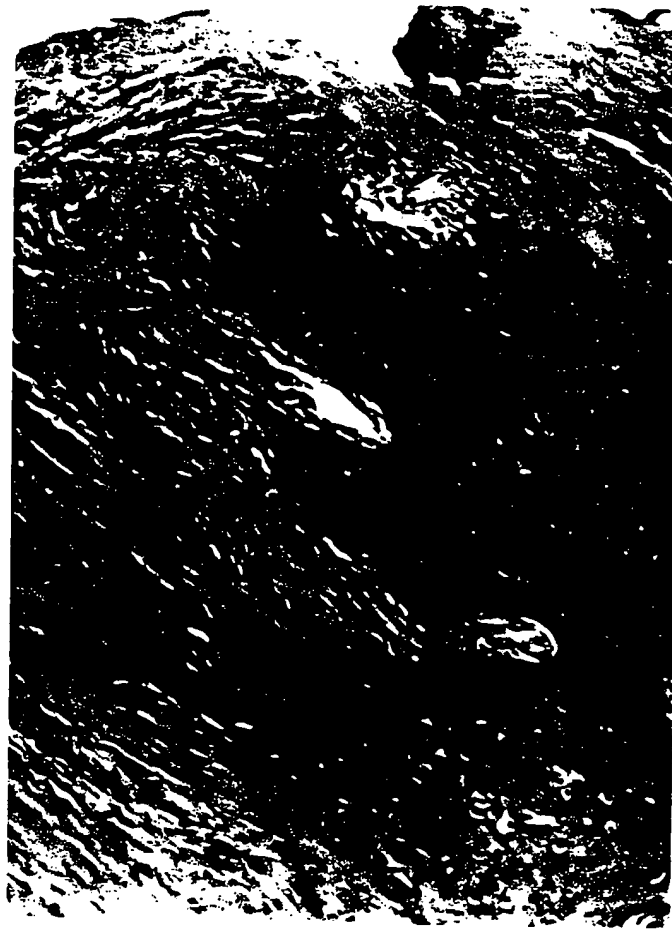


FIG. 7C

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FIG.8A

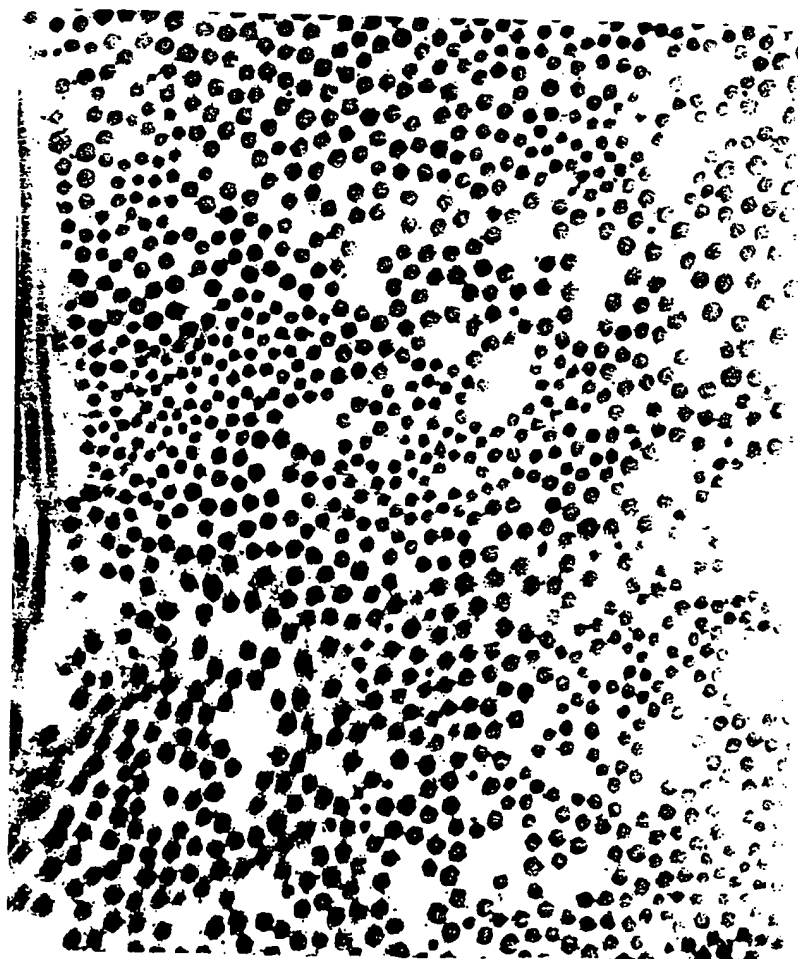


FIG.8B

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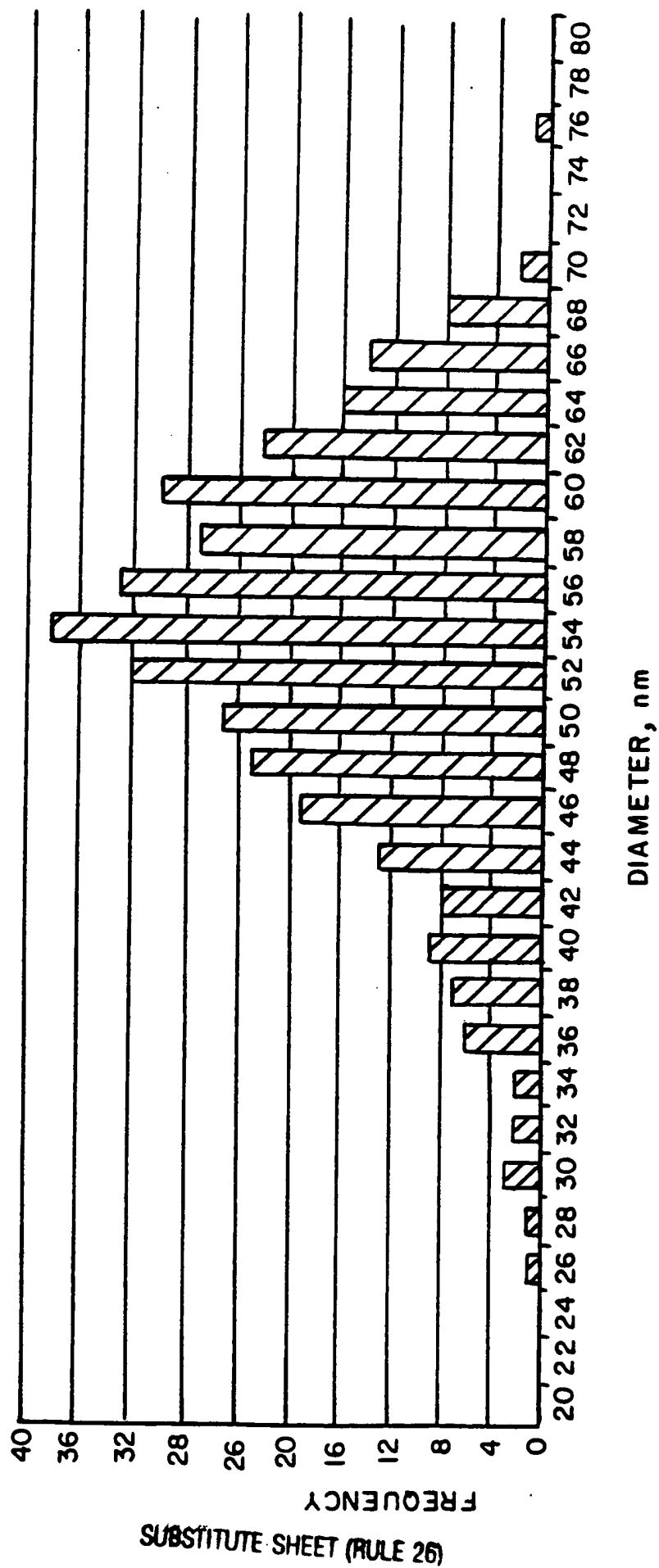


FIG. 9A

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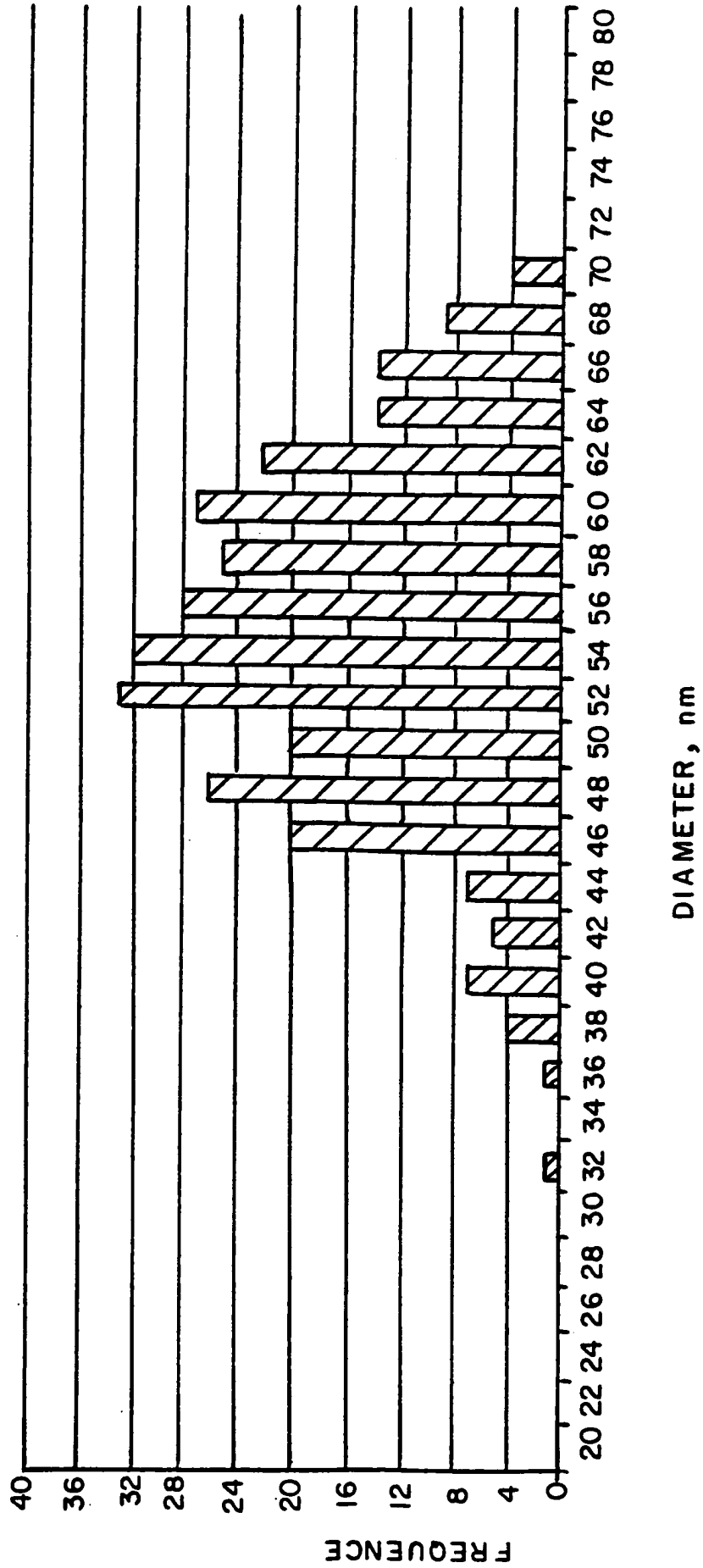


FIG. 9B



FIG. 10

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 A61L27/00 A61F2/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,4 097 234 (SOHDE TAKESHI ET AL) 27 June 1978 see column 2, line 15 - line 34; claims ---	1-23
X	WO,A,93 06791 (ORGANOGENESIS INC) 15 April 1993 cited in the application see the whole document ---	1-23
X	US,A,2 637 321 (ARTHUR CRESSWELL) 5 May 1953 see column 1, line 41 - line 55; claims ---	1-4
X	GB,A,831 124 (ALFRED BLOCH & IRVING BERNT ONESON) 23 March 1960 see claims; examples I-IV ---	1
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

28 June 1995

Date of mailing of the international search report

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Authorized officer

ESPINOSA, M

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A,3 433 864 (HIGHBERGER JOHN H ET AL) 18 March 1969 see claims; examples I-II ---	1
A	FR,A,1 385 668 (ETHICON, INC.) 7 December 1964 see claims ---	1
A	GB,A,1 108 331 (ETHICON, INC.) 3 April 1968 see claims; examples ---	1
A	WO,A,92 18172 (COLETICA) 29 October 1992 see claims ---	1
A	US,A,5 171 273 (SILVER FREDERICK H ET AL) 15 December 1992 cited in the application see claims ---	1
A	US,A,5 263 984 (LI SHU-TUNG ET AL) 23 November 1993 cited in the application see claims; figures -----	1

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 20-21, 23
because they relate to subject matter not required to be searched by this Authority, namely:
Remark - Although claims 20-21, 23 are directed to a method of treatment of the human or animal body the search has been carried out and based on the alleged effect of the composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4097234	27-06-78	NONE	
WO-A-9306791	15-04-93	US-A- 5378469 EP-A- 0607346 JP-T- 7500037	03-01-95 27-07-94 05-01-95
US-A-2637321	05-05-53	BE-A- 477545 CH-A- 272100 DE-C- 911322 FR-A- 955840 GB-A- 606427	20-01-50
GB-A-831124		NONE	
US-A-3433864	18-03-69	DE-A- 1720192 FR-A- 1515365 GB-A- 1175622 NL-A- 6703536	03-06-71 12-06-68 23-12-69 11-09-67
FR-A-1385668	05-05-65	DE-A- 1469075	19-12-68
GB-A-1108331		NONE	
WO-A-9218172	29-10-92	FR-A- 2675048 AU-A- 1668392	16-10-92 17-11-92
US-A-5171273	15-12-92	NONE	
US-A-5263984	23-11-93	US-A- 4880429 US-A- 5007934 US-A- 5116374 AU-B- 4048493 CA-A- 2134111 EP-A- 0639959 WO-A- 9321857 AU-B- 605723 AU-A- 2424588 DE-A- 3879921 DE-T- 3879921 EP-A,B 0324852	14-11-89 16-04-91 26-05-92 29-11-93 11-11-93 01-03-95 11-11-93 17-01-91 13-02-89 06-05-93 14-10-93 26-07-89

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5263984		JP-T- 2500654	08-03-90
		WO-A- 8900413	26-01-89
		US-A- 5158574	27-10-92
		US-A- 5306311	26-04-94
		US-A- 5258043	02-11-93
		AU-B- 637605	03-06-93
		AU-A- 5337990	26-09-90
		CA-A- 2050471	03-09-90
		EP-A- 0461201	18-12-91
		JP-T- 4504968	03-09-92
		WO-A- 9009769	07-09-90
		US-A- 5108438	28-04-92
